BIOCHEMICAL AND HEMATOLOGICAL RESPONSES OF THE BANDED KNIFE FISH *Gymnotus carapo* (LINNAEUS, 1758) EXPOSED TO ENVIRONMENTAL HYPOXIA

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ABSTRACT

Oxygen of tropical freshwater environments fluctuates drastically. Eutrophic lakes and ponds of warm waters frequently reach very low oxygen concentrations. This is the most common habitat of the banded knife fish "tuvira" *Gymnotus carapo*. This electric fish is reported to present bimodal breathing to cope with low environmental oxygen. Biochemical responses can be also observed in fishes facing hypoxia but none were studied in tuvira. In the present study, haematological and metabolic changes were investigated in two groups of fish exposed to hypoxia for 1 and 3 hours. Haematocrit, red blood cells and haemoglobin concentration indicated erythrocyte release from hematopoietic organs and swelling of red blood cells. Glycogen, glucose, lactate, pyruvate, and amino acids were quantified in liver, kidney and white muscle. The metabolic profile of *G. carapo* to cope with hypoxia suggested liver gluconeogenesis probably supported by proteolysis. The kidney and liver presented the same biochemical trend suggesting similar metabolic role for both organs. Glucogenolysis followed by glucose fermentation and protein mobilisation was observed in the white muscle. The air breathing behaviour of tuvira works in parallel with metabolism to prevent damages from hypoxia. Metabolic adjustments are observed when the air taking is avoided.

Key words: hypoxia, biochemical adaptation, *Gymnotus carapo,* metabolism, fish.

RESUMO

Respostas bioquímicas e hematológicas de tuvira *Gymnotus carapo* (Linnaeus, 1758) exposta à hipóxia ambiental

O oxigênio de água doce dos ambientes tropicais flutua drasticamente. Lagos e lagoas eutróficos de ambientes temperados freqüentemente atingem baixas concentrações de oxigênio. Este é o habitat mais comum da tuvira *Gymnotus carapo*. Neste peixe elétrico é descrita a respiração bimodal para enfrentar baixos níveis de oxigênio. Em peixes, as respostas bioquímicas também podem ser observadas, mas nenhuma foi estudada em tuvira. Neste estudo foram investigadas as alterações hematológicas e metabólicas em dois grupos de peixes expostos à hipóxia por 1 e 3 horas. O hematócrito, os eritrócitos e a concentração de hemoglobina indicaram liberação de hemácias por órgãos hematopoiéticos e intumescimento celular. Glicogênio, glicose, lactato, piruvato e aminoácidos foram quantificados no fígado, no rim e em músculo branco. O perfil metabólico de *G. carapo* para enfrentar a hipóxia sugeriu neoglicogênese hepática por proteólise. O rim e o fígado

apresentaram a mesma tendência metabólica, sugerindo o mesmo papel metabólico de ambos os órgãos em hipóxia. Foi observada neoglicogênese em músculo branco, seguida da fermentação de glicose e da mobilização protéica. O comportamento de respiração área em tuvira funciona paralelamente ao metabolismo adaptativo para prevenir os danos da hipóxia. Os ajustes metabólicos foram observados a partir do momento em que a tomada de ar foi bloqueada.

Palavras-chave: hipóxia, adaptação bioquímica, *Gymnotus carapo*, metabolismo, peixes.

INTRODUCTION

Low oxygen environments are usually found in many tropical plain lakes, ponds, swamps and other eutrophic waters (Almeida-Val *et al.*, 1993). Animals living in those conditions must preserve any strategy to cope with hypoxia. As the oxygen decreases due to any factor, fishes usually respond by escaping to other environments. However, if hypoxia is unavoidable, other mechanisms must be triggered in order to survive. Amongst the large set of organic strategies as hyperventilation, bradicardia, cardio-respiratory synchronism and peripheral vascular constriction, the biochemical responses contribute to face low oxygen levels, buffering their effects.

Many typical biochemical responses to environmental low oxygen are reported in fishes. The glycogen bulk enhancement is very common particularly in the liver (Johnson, 1975; Philips & Hird, 1977; Renauds & Moon, 1980; Moose, 1980; Moraes *et al.,* 1996; Moraes *et al.*, 1998). Glucose fermentation yielding lactate is also reported (Jorgensen & Mustafa, 1980; Dunn & Hochachka, 1986; Yu & Woo, 1987). Some species export large amounts of muscle lactate to plasma (lactate releasers) while others (non-releasers) keep lactate within muscle cells. Reduction of metabolic rate under hypoxia is another biochemical response. It is often reported and is usually named metabolic depression. Disregarding the biochemical responses, the behavioural and/or physiological strategies to cope with hypoxia are observed in many species to preserve the cell energetic efficiency.

The banded knife fish (tuvira) *Gymnotus carapo* L. is ordinarily found in very low oxygen environments (Crampton, 1998). This teleost belongs to a group that includes about one hundred species and nearly thirty genera. This Gymnotiforme is an electric fish, presents bimodal breathing, and the mechanisms of air intake and partitioning are well studied and reported (Liem *et al.* (1984). As the access to air is prevented, the total oxygen consumption of tuvira drops drastically to 69% and the water O_2 extraction increases about 275% (Liem *et al.,* 1984). Considering the behavioural and physiological strategies to cope with hypoxia, we questioned the metabolic responses. Does tuvira present any biochemical mechanism working in addition to those others? Is the bimodal breathing behaviour an accessory or a cardinal strategy for the species? The present paper reports the metabolic changes observed in tuvira under severe environmental hypoxia, avoiding the air breathing strategy, and considering the role of both responses.

MATERIALS AND METHODS

Tuviras (*G. carapo*) were captured in Monjolinho River (22°01'S-47°53'W) Sao Carlos, Brazil, April 1999. The animals were adult fish but living off their reproductive period. Following the capture, they were held in a covered indoor tank in aerated water and temperature of 25°C ± 1.5 for seven days.

Experimental design

Eighteen fish were randomly netted from the indoor tank and quickly transferred for the tests into 15-liters glass aquaria with a constant flow of aerated, tap water. The fish were separated into four experimental lots (I-II-III and IV) with six animals each, keeping the average of 25g of fish per liter of water. The animals were kept under normoxia (7 mgO_2/ml) at 25°C ± 1.5 for 24 hours. After this period, lots II and III were subjected to hypoxia (1 mgO_2/ml); the former for 1 hour and the latter for 3 hours. Hypoxia was induced by disconnection of the aeration system and stopping of the water flow. The fish were kept avoided to reach the surface by a plastic grid fitted one-inch

below the water surface to prevent bimodal breathing. The plastic grid was adapted at the moment the fish were transported to the aquaria, 24 hours before starting the hypoxia. Lots I and IV were used as control for II and III respectively. The animals of the control groups were treated in the same way as those of the lots II and III, except by the aeration and water flow, which were normally kept. After exposure to hypoxia, the fish were quickly removed from the aquaria and blood samples were withdrawn from caudal veins into heparinized syringes for taking of haematological and biochemical parameters. The animals, previously anaesthetised by MS222, were killed by a blow on the head, followed by pinching of the spinal cord. White muscle, liver and kidney were excised and immediately transferred to liquid nitrogen for subsequent analysis of metabolic intermediates.

Blood

Red blood cells (RBC) were counted in a Neubauer chamber; total haemoglobin (Hb) was determined based on its complete conversion into cyanmetahemoglobin read at 540 nm, and hematocrit (Ht) was read after centrifugation as usual. The blood samples were centrifuged at 12,000 g for 3 min at room temperature and plasma (100 µl) was deproteinized by 20% trichloroacetic acid-TCA (900 µl). Protein free plasma samples were centrifuged at 12,000 g for 3 min at room temperature. The supernatants were used for glucose, pyruvate, lactate and ammonia determination.

Biochemical analysis

The excised organs (100 mg) were defrosted, mechanically disrupted with a Potter Evelhjein homogenator by two 15-seconds strokes into 20% TCA (900 µl). The free protein extracts were centrifuged at 12,000 g for three min at 5°C and the supernatants were used to evaluate metabolite concentration. Plasma metabolites were estimated by the following end-point methods: pyruvate by 2,4-dinitrophenilhydrazine adapted from Lu (1939), lactate by p-phenilphenol (Harrower & Brown, 1972), ammonia by Nessler's method modified by Gentzkow & Masen (1942), free amino acids by ninhydrin adapted from Copley (1941), and free reducing sugars, assumed as glucose, by phenol sulphuric-acid (Dubois *et al.,* 1956). The glycogen determination was modified from Bidinotto *et al.* (1997) as follows. After alkaline digestion of 100-200 mg of tissue per ml of 6 N KOH under a boiling water-bath, 100 µl of extract were transferred to 3.0 ml of ethanol and 250 µl of saturated K_2SO_4 was added. The samples were centrifuged at 3,000 g for 3 min at room temperature. The supernatant was discarded and the pellet re-suspended into distilled water. The carbohydrate content was determined into suitable aliquots by Dubois' method (1956).

The chemicals used as standard were analytical grade, purchased from Sigma Chemical Co. St. Louis, Mo or Merck. The MS222 was from Sandoz. All other reagents were analytical grade.

Significant differences were established by the non-parametric test of Kruskal-Wallis and the significance level was set at $p < 0.05$.

RESULTS

Tuvira subjected to experimental conditions sought frequently the water surface. This is a typical behaviour of the species in low aerated water. The hematocrit of the hypoxic animals increased significantly ($p < 0.01$). The red blood cell number and the haemoglobin concentration increased at the first hour of hypoxia, decreasing after three hours (Fig. 1).

The tissue metabolite levels suggested a particular outline under environmental hypoxia. The hepatic glycogen concentration increased from 29.97 to 60.94 µmols of glucosyl-glucose per gram of wet tissue. The kidney trend was similar, but in white muscle glycogen slightly decreased from 15.67 to 9.8 µmols of glucosyl-glucose per gram of wet tissue (Fig. 2a). The kidney and liver contents of glucose increased progressively with hypoxia. However, white muscle glucose decreased from 15.67 to 9.8 µmols per gram of tissue (Fig. 2b). A striking raise of lactate from 2.9 to 8.1 µmols per gram of wet tissue was observed in the liver and a considerable increase from 4.5 to 8.8 µmols per gram of wet tissue was observed in the white muscle during the first hour of hypoxia (Fig. 2c). However, those values returned to basal levels after three hours.

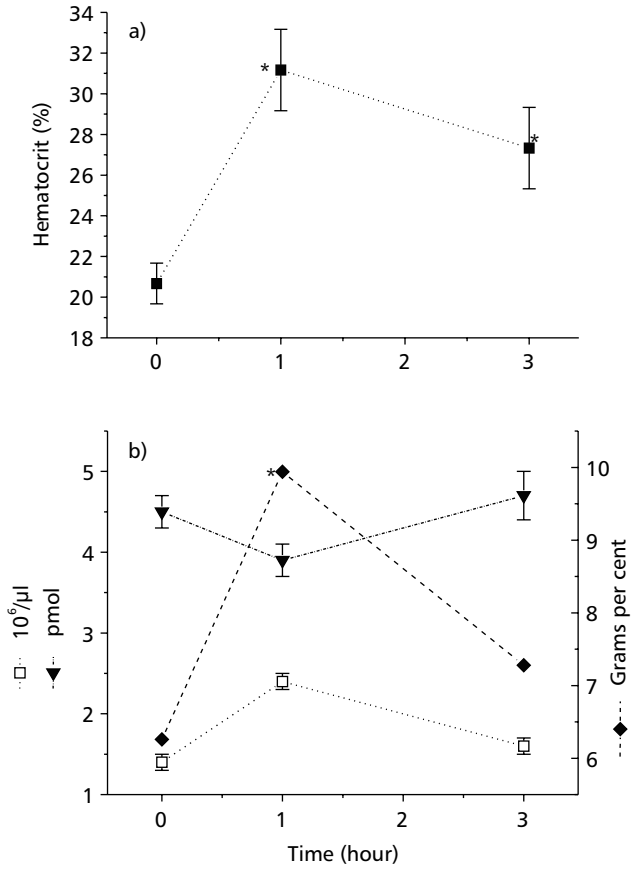

Fig. 1 — Hematological parameters of the banded knife fish tuvira (*G. carapo*), exposed to hypoxia (1 mgO_2/ml) for 1 and 3 hours. (a) Hematocrit percent (■). (b) Total hemoglobin (Hb) grams percent (◆), red blood cells (RBC) x 10^6 μl^{-1} (□), and mean cellular hemoglobin content (MCHb) pmol (▼).

A similar trend was observed in pyruvate concentrations of liver, white muscle and kidney under hypoxia (Fig. 3a). Amino acids exhibited the same tendency in the liver, white muscle and kidney.

A significant increase was observed in the first hour of hypoxia, tending to return to initial values after three hours (Fig. 3b). Plasma levels of lactate rose from 1.38 to 3.83 µmol per gram of wet tissue, but glucose remained constant (Table 1). Ammonia concentration enhanced in liver, white muscle and kidney but did not change in the plasma (Table 2).

DISCUSSION

Low oxygen is usually a very common stressor to water living organisms from tropical environments. As many other environmental factors, it is also accountable for a set of organic changes. Several species besides *G. carapo* have been reported as responsive to environmental oxygen concentration, particularly in terms of blood parameter variations (Swift, 1981; Moraes *et al.*, 1996, 1998). Blood responses, prior to many others, are considered fundamental to internal adjustments to cope with a number of stressors.

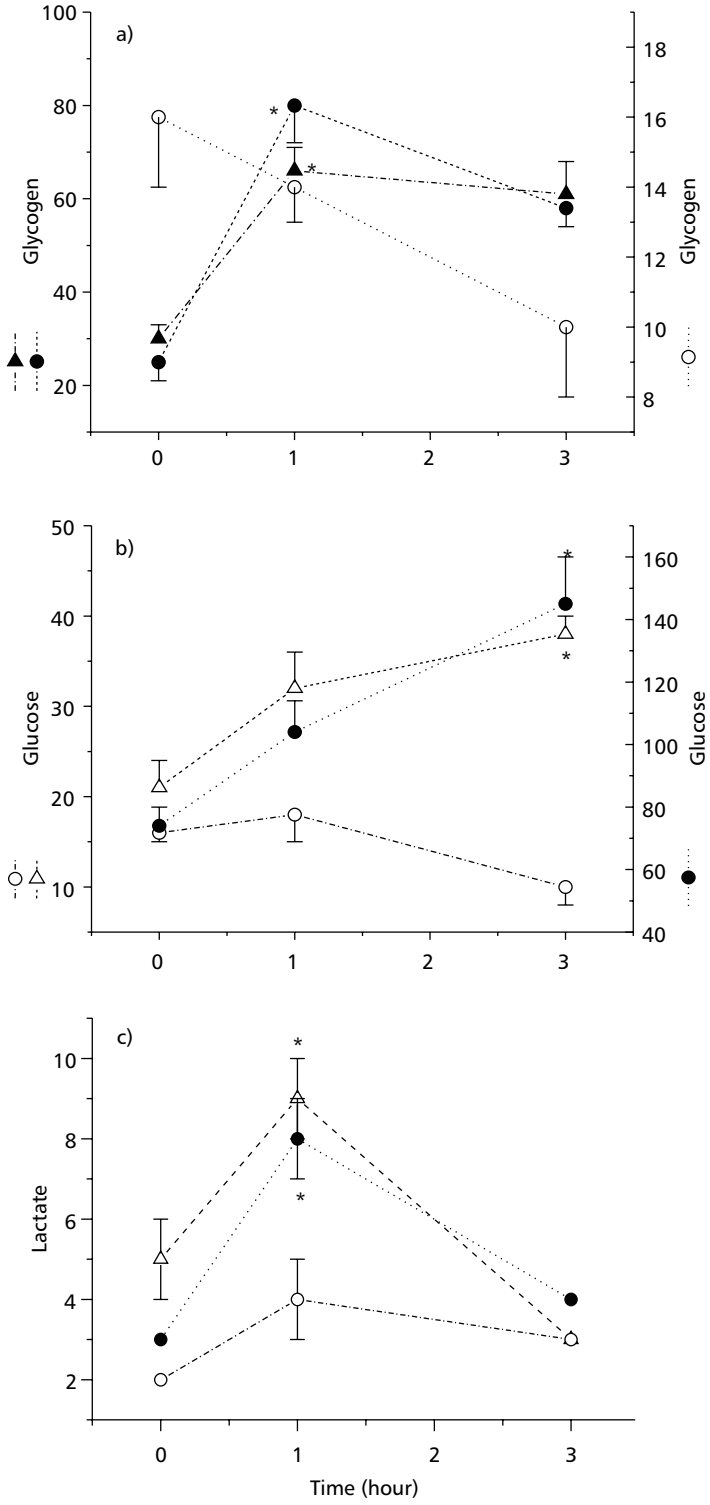

Fig. 2 — Glycogen (a), glucose (b) and lactate (c) contents in liver (●), white muscle (○) and kidney (△) of the banded knife fish tuvira (*G. carapo*), exposed to hypoxia (1 mgO_2/ml) for 1 and 3 hours are expressed in µmol per gram of wet tissue. The mark (*) means significant difference compared to control at the level of $p < 0.05$.

Among that, increase of hematocrit is usually observed as the result of erythrocyte swelling, decrease of plasma volume, increase of red blood cell number or a combination of such factors (Peterson, 1990). Tuvira presented the usual blood response to hypoxia, i.e., a hematocrit increasing of about 50%. However, cell haemoglobin concentration (MCHb) lessened at the first hour of hypoxia followed by the RBC enhances. These data suggest both erythrocyte release from haematopoietic organs and swelling of red blood cells. Subsequently, the number of red cells tended to normality reaching the initial values, but the cell volume remained high. This response should be expected as adaptive considering that hypoxia-related stress rely on intra-erythrocyte modulators (Weber, 1982; Nikinmaa, 1990).

The fact that tuvira is an electric fish, the ability to detect the container walls and the grid at the water surface should be considered as a stressor. However, it also considered that the fish remained in tanks prior to the experiments for many days. Moreover, the set of organic changes observed comparing hypoxic with normoxic animals as the only imposed difference was the environmental oxygen.

Tissue metabolic adjustments belong to the group of secondary organismal defences against environmental stresses (Selye, 1973). Therefore, these responses are not so immediate as, for instance, cortisol release and the haematological consequences. The biochemical responses of organisms are an attempt to maintain the cell status. Some physical parameters, such as pH and redox potential, must be specially preserved to keep the cellular integrity (Hochachka, 1980). The absence or decrease of external oxygen signalises to the organism toward adopting the proper metabolic changes to preserve the life. Alterations of the metabolic profile in response to external changes have been reported from many species as rainbow trout (Dunnn & Hochachka, 1986), *Cyprinodon variegatus* and *Poecilia latipinna* (Peterson, 1990) and *Hoplias malabaricus* (Moraes *et al.,* 1996, 1998). The metabolic effects observed in tuvira were a set of significant responses to cope with environmental hypoxia. Considered the initial increase of hepatic lactate (twofold), the return to basal values, and the duplication of glucose, pyruvate and glycogen, we can state that the liver gluconeogenic mechanisms were triggered. Hepatic gluconeogenesis from lactate has been reported in many fish (Johnson, 1975; Philips & Hird, 1977; Renauds & Moon, 1980; Moose, 1980; Moraes *et al.,* 1996, 1998). It is interesting to see that liver amino acids and ammonia showed the same trend observed to lactate, suggesting that this gluconeogenesis was also supported by proteolysis. Similar tendency was reported for *H. malabaricus* submitted to environmental hypoxia (Moraes *et al.*, 1996) and to the functional one (Moraes *et al.*, 1998) caused by nitrite. The kidney and liver of tuvira depicted comparable trends insofar as the intermediate metabolism is concerned, suggesting that these organs adopted akin, metabolic behaviour to cope with hypoxia. Little data are available concerning renal metabolism under hypoxia, but *H. malabaricus* reportedly showed a similar metabolic profile (Moraes *et al.*, 1996). In addition, Jorgensen & Mustafa (1980) have reported renal lactic fermentation of *Platichthys flesus* under hypoxia.

White muscle from *P. flesus* (Jorgensen & Mustafa, 1980), *Channa maculata* (Yu & Woo, 1987) and rainbow trout (Dunn & Hochachka, 1986) consume glucose producing lactate. Differently, species as *Carassius auratus* (Van der Thillart *et al.*, 1980), *Symbranchus marmoratus* (Almeida-Val *et al.*, 1993) and *Hoplias malabaricus* (Moraes *et al.,* 1996, 1998) drove gluconeogenesis under hypoxia. The white muscle glucose and pyruvate remained constant although the glycogen bulk declined. This picture is very suggestive that the choice to supply the muscle energetic demands under hypoxia was the breakdown of glycogen. This response has been reported among fishes (Moraes, 1997a, b; Dunn & Hochachka, 1986). Consume of glycogen was anaerobic, however the effect of lactic fermentation mechanisms were promptly compensated after an hour. The muscle lactate concentration was reverted after three hours probably as a consequence of the exportation to the plasma. The considerable increase of free amino acids and ammonia is suggestive of protein mobilisation either in addition to glucose fermentation by many tissues or in the synthesis of new proteins to adjust the cell machinery.

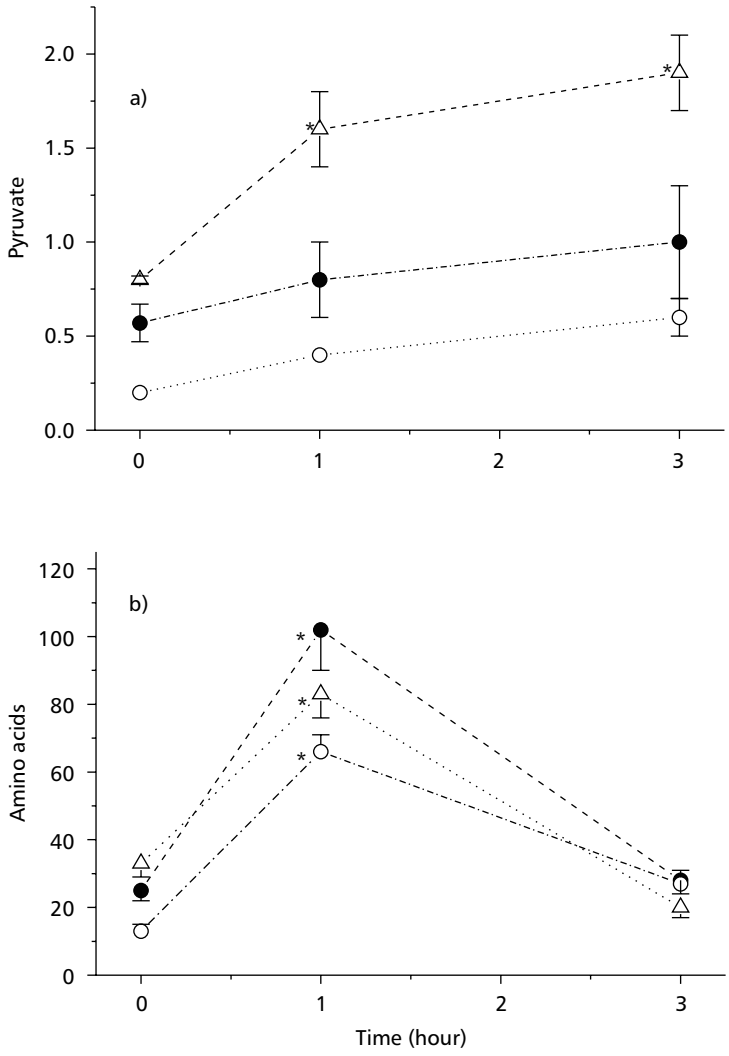

Fig. 3 — Pyruvate (a) and free amino acids (b) in liver (●), white muscle (○) and kidney (△) of the banded knife fish tuvira (*G. carapo*), exposed to hypoxia (1 mgO_2/ml) for 1 and 3 hours, are expressed in µmol per gram wet-tissue. The mark (*) means significant difference compared to control at the level of $p < 0.05$.

We have observed the metabolic changes in freshwater fishes to cope with tropical usual environmental stressors, such as hypoxia. Strategies against it may be visible or silent responses. Considering the usual air-breathing behaviour of tuvira we questioned about the presence of biochemical responses as a silent strategy. In view of the span of the observed metabolic adjustments to hypoxia, their magnitude during the first hour, and the changes of the haematological parameters, led us to consider that the set of internal responses is pivotal. Tuvira seems to be not a lactate producer but the metabolic interfaces it shares with species whose biochemical responses are decisive are meaningful. Therefore, we understand that the biochemical and haematological responses are equally vital in the rank of strategies that allow the banded knife fish tuvira to survive under hypoxia.

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